Self-assembly of complex structures is commonplace in biology but often poorly understood. In the case of the actin cytoskeleton, a great deal is known about the components that comprise higher order structures, such as lamellar meshes, filopodial bundles and stress fibers. Each of these cytoskeletal structures contains actin filaments and crosslinking proteins, but the role of crosslinking proteins in the initial steps of structure formation has not been clearly elucidated. We employ an optical trapping assay to investigate the behaviors of two actin crosslinking proteins, fascin and alpha-actinin, during the first steps of structure assembly. Here we show that these proteins have distinct binding characteristics that cause them to recognize and crosslink filaments that are arranged with specific geometries. Alpha-actinin is a promiscuous crosslinker, linking filaments over all angles. It retains this flexibility after crosslinks are formed, maintaining connection even when the link is rotated. Conversely, fascin is extremely selective, only crosslinking filaments in a parallel orientation. Surprisingly, bundles formed by either protein are extremely stable, persisting for over 0.5 h in a continuous wash. However, using fluorescence recovery after photobleaching (FRAP) and fluorescence decay experiments we find that the stable fascin population can be rapidly competed away by free fascin. We present a simple avidity model for this crosslink dissociation behavior. Together, these results place constraints on how cytoskeletal structures assemble, organize, and disassemble in vivo.

The actin cytoskeleton forms and manages an array of diverse structures with regularity and precision. The same set of tools is used by all cells to many different ends: for example, muscle cells form sarcomeres while non-muscle cells form filopodia, lamellipodia, and stress-fibers (1-8). Formation and maintenance of actin cytoskeletal structures is critical for proper cell functions and viability (9-16) but the mechanisms of these actions are poorly understood.

A great deal of work has been done examining how cytoskeletal proteins are regulated, and how they are sorted within the cell. While assembly of complex cytoskeletal structures is clearly essential for the proper mechanical behavior of the cell, the physical mechanisms driving their assembly are incompletely understood. Mechanisms have been proposed for formations of structures such as filopodia (17-19) and stress-fibers (20, 21). Filopodial nucleation is thought to occur by formation of the filopodial tip complex bringing together actin filament barbed ends, allowing local elongation leading to filopodial growth (17). A recent study using electron tomography has revealed that prior to filopodia nucleation at the plasma membrane of cultured fibroblasts, filaments in the lamellipodia can be observed in distinct pairs (22). The authors suggest these pairs may play a key role in filopodial nucleation. Stress fiber formation is proposed to function through the coalescence of actin bundles mediated by myosin II (23). Both of these key cellular structures require actin crosslinking proteins. In these models the crosslinking proteins are described as simple molecular staples, but it is possible that they play a critical role in the formation of these and other high order structures. Here we attempt to gain insight in the formation of cytoskeletal structures by performing an in vitro analysis of two very distinct actin crosslinking proteins, fascin and smooth muscle alpha-actinin to determine orientations of filaments that are required to allow these proteins to form crosslinks.

Alpha-actinin is a member of the spectrin family of proteins and if found in all eukaryotes (24). It is functional as an anti-parallel homodimer. Each monomer is comprised of two
carboxy-terminal EF hand domains, four spectrin repeats, and two amino-terminal calponin homology (CH) domains that comprise the actin binding domain (ABD) (25) (Fig. 1A). The actin binding domain is conserved with other actin crosslinkers including fimbrin and filamin (25). Tissue specific isoforms of alpha-actinin are involved in z-discs of sarcomeres in muscle cells and stress-fibers in non-muscle cells (24). In vitro experiments have shown that alpha-actinin can form homogeneous actin networks (three-dimensional meshworks with uniform crosslinker density and mesh size), tight bundles, or a mixture of the two (26-29). Electron microscopy has captured alpha-actinin interacting with actin filaments in multiple orientations: crosslinking parallel filaments, anti-parallel filaments and side binding both actin binding domains of a single alpha-actinin dimer to one filament (30-32).

Fascin is a small globular protein that consists of four fascin-repeat domains tightly packed together (Fig. 1B). A crystal structure has been available for some time** and now new structures and examinations are giving more insight into the location of the actin binding interfaces on the molecule (22,33,34). Fascin is conserved from Drosophila to humans, and is a major component of the finger-like cellular projections known as filopodia, where it crosslinks the tight actin bundle at the filopodial core (35). The actin in these bundles is arranged in a parallel manner with the barbed ends towards the tip of the projection (17). Fascin has not been observed to be a stable component of non-bundling structures (e.g., meshworks), instead forming a ‘network of bundles’ rather than a homogenous network as seen in alpha-actinin (36,37). In vivo and in vitro work has shown fascin to be a dynamic crosslinker, rapidly turning over within filopodial bundles (38,39).

Direct manipulation of actin crosslinking proteins has been performed (40,41). Using optical trapping techniques, Miyata et al. report rupture forces and bond lifetimes of alpha-actinin to actin bonds. Ferrer et al. report rupturing the bonds and observing bond lifetimes between actin and the crosslinking proteins alpha-actinin and filamin. These studies show that the alpha-actinin to actin bond can sustain a large force (40-80 pN) and report average bond lifetimes of between 2.5 and 20 seconds. These are some of the first single molecule studies of crosslinkers, and they display surprising bond strength and stability.

Current models of how cytoskeletal structures nucleate and grow are based largely on evidence from video microscopy and snapshots from electron micrographs (17,20,22,38). These studies make the assumption that growing filaments must attain a specific orientation and proximity before crosslinking proteins can nucleate and stabilize structures. In our study we test that assumption directly by recreating a variety of actin structures like those crosslinking proteins might encounter in cells, and assess the ability of the proteins to form and maintain crosslinks. Alpha-actin and fascin show strikingly different behaviors in this assay. Alpha-actinin crosslinks in every structure tested, while fascin only crosslinks filaments in one specific orientation. Once the parameters for interaction were determined we examined the dissociation behavior of these proteins from actin bundles and found that fascin dissociation from bundles seems to happen primarily in the presence of competitive agents but not in isolation. We propose a model where crosslinks rapidly toggle between being bound to one and two filaments but are rarely lost to solution unless a competitive agent is present.

**EXPERIMENTAL PROCEDURES**

Protein Purifications- Actin was purified using an established protocol (42). Actin was polymerized at a concentration of 10 μM monomer in assay buffer (AB) (25mM imidazole, pH 7.5, 25 mM KCl, 1 mM EGTA, 4 mM MgCl2 and 10 mM DTT) in the presence of 2 mM ATP using 90% dark (unlabeled) actin and 10% biotinylated actin, and then stabilized with Tetramethylrhodamine (TMR) -phalloidin. Polarity labeled actin was made by growing a cap consisting of 30% TMR-labeled actin and 70% dark actin on the barbed end of dark filaments then coating with alexa-633 phalloidin. Polarity labeled actin was made by growing a cap consisting of 30% TMR-labeled actin and 70% dark actin on the barbed end of dark filaments then coating with alexa-633 phalloidin. Human fascin was purified using an established method (43). Chicken smooth muscle alpha-actinin was purified using an established method (44). An additional gel filtration step was performed over a Sepharose 4B (Sigma-Aldrich) column to remove contaminants (Fig. S1). Proteins with chemical modifications (Biotin, TMR, and Atto-647N) were labeled using maleimide chemistry. Reactions of protein and 2 to 10 times
molar excess dye were incubated overnight at 4°C in PBS pH 7.0. Excess dye was removed by at least two hour incubation with Biobeads (Biorad) and then overnight dialysis into storage buffers, including two buffer exchanges.

**Optical Trapping Assays-** To make neutravidin coated beads for use in these experiments, biotinylated polystyrene beads (1 μm diameter, Molecular Probes) were rinsed three times in PBS, then incubated for one hour at room temperature with 2.5 mg/mL neutravidin (Molecular Probes). The beads were then incubated with 10 mg/ml BSA or TMR-BSA for 30 seconds before being rinsed ten times in AB with 1 mg/ml BSA to complete blocking and washing. All experiments were performed on a home-built optical trapping and multi-color fluorescence microscope. Four-input laminar flow chambers coupled with fluid reservoirs and valves were used in all trapping assays (45). All solutions were prepared in AB. Flow chambers were blocked with 1mg/ml BSA. Reservoirs were loaded with solutions. Reservoir one was loaded with 4 μL neutravidin coated beads in 1 mL observation buffer (AB plus 0.86 mg/mL glucose oxidase, 0.14 mg/mL catalase, 9 mg/mL glucose). Reservoir two was loaded with 1 μL of 10 μM F-actin diluted in 1 mL observation buffer. Reservoir three was observation buffer only. Reservoir four contained a final concentration of 1μM crosslinking protein in observation buffer for angular dependence assays and 0.1 μM crosslinking protein when making bundles for unzipping experiments. Solutions were allowed to flow through the flow chamber, creating four distinct lanes in the main channel. One to four beads were trapped in the first lane. The beads were transited through the actin channel where actin filaments were allowed to stick to the beads. The beads and actin were arranged into the desired geometry in the buffer only lane. Movie acquisition was started then the bead and actin structures were moved into the crosslinking protein lane. Movies were recorded using Andor Luca and iXon cameras with epifluorescence illumination, at frame rates from 0.1 to 0.2 seconds per exposure. Data was analyzed using ImageJ movie processing and angle measurement tools.

**Fluorescence and FRAP-** Fascin-actin bundles were made by incubating 10 μM F-actin (10% biotin, 90% dark) with 4 μM labeled atto-647N-fascin in AB for two hours. Flowcells were formed using double-sided tape to adhere 24 x 60 mm coverslips crosswise on the microscope slides. Chambers were loaded with neutravidin solution (0.5 mg/ml) and incubated for two minutes, then blocked with a 1 mg/ml BSA in AB for ten minutes, before loading bundle solution (diluted to 0.5 μM actin in AB) and incubating for 2 minutes. Observation buffer was then loaded and the slide was placed on the microscope. Solution changes were performed by spotting solutions at the opening of the flow chamber and wicking them through with filter paper. Before each assay the chamber was washed with an additional observation buffer wash to remove any free fascin from solution. FRAP was performed by closing down the field iris until only the area to be bleached was visible, then increasing the laser power for the bleaching step. Optics were restored to observation settings and movies were recorded. Crosslinking protein washes were performed using 3 μM protein in observation buffer.

**RESULTS**

We set out to determine whether alpha-actinin and fascin could bind filaments in any orientation presented or if they would only form crosslinks when presented with filaments already arranged in a specific orientation. Actin geometries of desired specifications were arranged using an optical trapping microscope coupled to a flow chamber (Fig. 1C) (45). Once the desired geometries (Fig. 1D-J) were constructed they were moved into a flow lane containing 1 μM crosslinking protein (Fig. 1C). Crosslinking events were counted using a binary link/no link metric. Movies were acquired at rates of 5-10 frames per second. Filaments had to remain together in a fixed orientation for at least three consecutive frames for a crosslinking event to be counted. When the filament polarity was known or could be deduced, events were recorded with specific angles. When the polarity was not known, binding events were categorized in pairs that contain both possible angles (e.g., 45/135°). The binding profile from these data is the angular dependence of binding. No crosslinking events or filament self-association was ever observed in the absence of crosslinking protein.
Alpha-actinin is a Promiscuous, Flexible Crosslinker - Alpha-actinin proved to be a promiscuous crosslinker (Fig. 2, Table 1). When parallel or anti-parallel filaments were tested, the filaments formed many crosslinks, like a zipper closing, forming bundles (Fig. 2D-H). When pairs of filaments crossed over a range of angles from 16-165° were probed, in all cases a single point of attachment was observed. These crosslinks formed within a few seconds of entering the crosslinking channel but the time scale cannot be precisely quantified with this system.

Once formed, these crosslinks were very stable. They were never seen to dissociate during the time of observation (2-4 minutes for single points of attachment in the presence of alpha-actinin). The stability of single links may be explained in two ways. One, it is possible there are two or more crosslinking proteins present at the linkage site (Fig. S2). Two, because the links are under some load, they may not be able to dissociate in the same manner as unloaded links (as suggested by Ferreri et. al (40)). Interestingly, these links remained bound even when the orientation of the filaments was changed and the linkage was rotated (Fig. 2I-P). This implies that the alpha-actinin remains flexible even while engaged in an active crosslink. Our observation period ended when the actin filaments broke or photobleached, or when the structure fell out of the optical traps.

Fascin Selectively Crosslinks Parallel Filaments - In contrast to alpha-actinin, fascin is an orientation-selective crosslinker. Fascin only crosslinked filaments when they were arranged in a parallel orientation (Fig. 3, Table 2). Anti-parallel filaments (Fig. 3J-N) and crossed filaments (Fig. 3A-J) showed no sign of fascin induced linkage. We estimate that transient crosslinks, if present, should have been observed in our system. Fascin has been shown by FRAP to dissociate at a rate of once per six seconds (.12 per second)(38), twenty fold slower than our detection threshold. Therefore, we are quite confident that we recorded most of the events that took place. When linkages were observed using the parallel orientation (Fig. 3O-S), the filaments invariably formed bundled sections (multiple links) and remained bound. Based on these data we believe it is unlikely that fascin will form stable crosslinks between two filaments oriented in anti-parallel or crossed orientations.

To support the polarity distinction observed in the fascin system, we performed a series of assays using polarity labeled actin (table 2). Rapid photobleaching of available far-red phalloidin labels (Alexa 633) made this a challenging experiment and limited the number of successful trials. In those instances the orientation of the filaments was recorded as soon as a filament was attached to a bead. The filaments were dramatically bleached before final geometries were attained. Tension between beads was used to establish intact filaments and the formation of crosslinks. Filaments arranged in a polar fashion rapidly formed bundles, while the filament pairs tested in an anti-parallel orientation did not form a bundle after approximately one minute of observation.

Two-Filament Bundles are Not Observed to Dissociate - Next we assessed the unzipping behavior of these two-filament bundles. Bundles were formed by attaching two filaments to a single bead then allowing them to crosslink in the presence of 0.1 μM alpha-actinin or 0.1 μM fascin. After being formed, bundles were moved into a flow lane containing only buffer, flow in all other lanes was stopped, and movies were recorded. After forty minutes of observation under continuous buffer flow neither the fascin nor alpha-actinin bundles were observed to dissociate (Fig. 4). This result was surprising given previous measurements of fascin dissociation rate (0.12 /sec) (38) and alpha-actinin actin binding domain dissociation rate (0.66 /sec) (27), and led us to further investigate the dynamics of the crosslinking proteins in bundles.

Dynamics of Fascin Within Bundles - A series of fluorescence recovery after photobleaching (FRAP) and fluorescence decay experiments reveal that fascin in bundles is stable and does not dissociate from the bundles unless competed away. Unfortunately, we were unable to prepare a functional, fluorescent-labeled alpha-actinin protein, so we restrict our discussion of dissociation to fascin.

First, bundles of dark biotinylated actin filaments and fluorescent atto-647N-fascin were made and washed into the neutravidin coated chamber (Fig. 5A). Two chamber volumes of buffer was then flowed in the chamber to remove
free fascin from solution. The fascin dissociation rate from the bundles was extremely slow, indistinguishable from background bleaching (Fig. 5E). When a section of a bundle was bleached with no fascin in solution, no recovery was seen, and the boundary between bleached and unbleached areas remained sharp, suggesting that the bound fascin does not appreciably redistribute within the bundle (Fig. 5A-E). This corroborates our results from the unbundling assay in the optical trap.

Since crosslinking proteins must bind to two filaments in order to form a link, we hypothesized that a high avidity might be dominating the behavior such that the multiple binding sites prevented complete dissociation. To test this bundles constructed of directly labeled fascin were rinsed with buffer containing unlabeled fascin. The fluorescence of the bundles was rapidly lost (decay of 0.10 s⁻¹) (Fig. 5F), matching closely the in vivo and in vitro results reported in Aratyn et al. (38) in which all experiments had free fascin in the cytoplasm or solution. Thus, crosslinks can be rapidly removed by competition.

The fascin replacement rate along the bundles is uniform (Fig. 5G). With no fascin in solution, a section of a bundle was bleached yielding a difference in fluorescence signal between the bleached and unbleached section. Labeled fascin was washed into a chamber and allowed to incubate for 30 seconds. The chamber was then washed with buffer to remove free fascin. After the wash the fluorescence intensity on the bundle inside and outside of the bleach spot was identical. This confirms that when fascin is present in solution, the replacement of fascin in the bundle takes place rapidly, further indicating that the presence of fascin in solution is critical for bundle crosslink turnover.

Interestingly, fascin is removed by other competitive agents. Bundles containing fluorescent fascin lost fluorescence extremely rapidly (0.254 s⁻¹) when unlabelled alpha-actinin was introduced (Fig. 5F).

**DISCUSSION**

We have directly observed that alpha-actinin is a promiscuous, flexible crosslinker with the ability to crosslink all orientations of actin filaments. Our data support a model where the actin binding domains on each end of the alpha-actinin dimer freely rotate with respect to each other, whether through flexible linker regions or a twist in the dimerization domain itself, and can bind any filament at any orientation that presents binding sites within a given interaction radius. This conclusion is well supported by other work. The diverse role of alpha-actinin in forming meshworks and bundles is well studied in vitro. Electron microscopy data has shown alpha-actinin has the ability to link filaments into parallel and anti-parallel bundles, as well as to side binding with both binding domains attached to the same filament (32). Rheology data has shown that it can form homogeneous networks (evenly distributed meshworks) (26,46). While crystallization of full-length alpha-actinin has proved difficult, electron microscopy coupled with partial crystal structures has yielded a great deal of insight into the structure of the functional dimer and how this molecule might function and supporting the idea that the binding domains can rotate with respect to each other (31,47-49).

We have also directly observed the binding behavior of fascin, which is a highly selective molecule. Fascin crosslinking is limited to filaments that have been arranged in a parallel orientation. This is consistent with known roles of fascin in formation of filopodial bundles and with in vitro observations (12,17).

The observation that no unbundling occurs in the absence of free crosslinking protein was surprising but not unprecedented. Fis, a DNA compaction and looping protein from E. coli, has been shown to condense (crosslink) DNA and remain stably bound in a buffer in the absence of protein for 20 minutes, but in the presence of competitive factors can be partially competed off of the DNA (50). Our competition assays show that similar behaviors may be present in both systems.

These results led us to the following model. Fascin that is involved in crosslinking (one fascin molecule interacting with two filaments) is very stable in the absence of competing free fascin. In tight fascin bundles filaments are arranged in in-register arrays that are very stiff on short length scales. When one fascin binding-site releases from an actin filament, the actin binding-site cannot diffuse away. For complete
dissociation to occur the second binding site on the fascin must release before the first one rebinds. Our results indicate that when a crosslinking fascin releases one binding site, it will rebind that site before the second site releases, leading to stable bundles when no fascin is in solution. This indicates that the on rate of the free actin-binding domain of the singly bound fascin is faster than the off rate of the bound actin-binding domain. We propose a model where the fascin molecule toggles back and forth between single and double bound states, with very rare dissociations (Fig. 6A).

When a competitive agent, such as free fascin molecules, is present in solution crosslinking fascin can be rapidly displaced from the bundle. When a bound fascin molecule enters the singly bound state, free protein in solution can compete for the transiently available binding site. Fascin that is bound to a single actin filament with no available second binding site (bound on the surface of the bundle or where another molecule is occupying the actin site) dissociates rapidly. This explains how all the fluorescent fascin is lost from bundles when dark fascin is introduced. Further, when two molecules compete for the same location in a bundle, as soon as one molecule dissociates the other can form a link, in many cases leading to crosslinker replacement or turnover (Fig. 6B). We also observed that free alpha-actinin in solution-displaced fascin in bundles more rapidly than solution fascin. It is possible that this rate difference is due to alpha-actinin having a higher affinity for actin than fascin, thus being a better competitor. It is also possible that, since alpha-actinin is a much longer molecule than fascin, alpha-actinin opens up fascin bundles as it integrates preventing singly bound fascin molecules from re-binding and causing a more rapid loss to solution.

Our fascin exchange results, closely match previously reported data, which was collected in the presence of free fascin in solution (in vitro and in vivo) (38,39). Our model begins to explain how bundles can be both stable and dynamic, allowing filopodia to form and bend without breaking, being overly rigid or overly soft.

Current Models of Actin Structure Formation- The orientation of actin filaments prior to structure nucleation is overlooked in most current models of cytoskeletal structure formation. The implicit assumptions are that the filaments grow in the correct orientation, align by thermal motion prior to assembly and/or align after crosslinks form. Here we have shown that different crosslinking proteins have different angular dependence of binding and that filament orientation is important for the earliest stages of structure formation and stabilization.

In the case of alpha-actinin, crosslinks can form regardless of the actin orientation but those crosslinks do not lock in an orientation. This has several ramifications for structure formation. Alpha-actinin can bind filaments if they are arranged correctly, or it can bind a pair of filaments and hold them in proximity to each other while a proper orientation is reached. Conversely, because it is so non-selective it may form links that are not beneficial. This would indicate that some form of regulation is required to prevent aberrant structure formation when alpha-actinin is involved. Alpha-actinin is known to have many different binding partners (51), and non-muscle alpha-actinin is calcium regulated (52) so it is reasonable to think active regulation likely prevents most aberrant activity.

Fascin is highly selective in its crosslinking behavior. Fascin localization in cells is mostly limited to filopodia and bundles that line the cell periphery. Based on our observations these filaments must be aligned before fascin can stabilize the structures. This finding supports the convergent elongation model of filopodial formation presented by the Borisy group (17), where filament ends come together and are linked by a filopodial tip complex into so called lambda precursors. Once the precursor forms the filaments can grow, and should be arranged in a parallel fashion, allowing fascin to bind. Other mechanisms may still prove valid. In cells there are also circumferential bundles that can cross each other in an anti-parallel orientation. These bundles may be realigned by some other means. For example, myosin X could crosslink the ends of the nascent bundles, leading to reorientation as they grow and the addition of fascin to stabilize a newly forming and growing filopodia (18,19). Fascin is known to have its binding behavior deactivated by phosphorylation (39), however the selective binding of fascin may aid in preventing fascin from forming unwanted structures, limiting
the amount of effort the cell must expend to regulate it.

There are many different actin crosslinking proteins. Some are found exclusively in bundles (e.g., fascin, fimbrin and espin) others almost exclusively in meshworks (e.g., filamin) and others in both types of structures (e.g., alpha-actinin). Our work shows that this selectivity may be due to inherent properties of the crosslinking proteins rather than, or in conjunction with, external localization and organizational cues. Understanding the properties of crosslinking proteins will help us place constraints on the behavior of the cytoskeleton, leading to a more complete understanding of cytoskeletal assembly and organization. The interactions probed here are complex and many questions still remain. It is possible that filament tension or twist may change crosslink behavior, as might other interacting proteins. In this study we have directly observed the behavior of two crosslinking proteins and established a road map for further testing the behavior of these types of proteins.

**FOOTNOTES**

* We would like to thank Gary Borisy for providing the fascin cDNA, and Yvonne Aratyn for helpful discussion. This work was funded by an American Heart Association predoctoral fellowship (to D.S.C.), and by NIH grant GM078450 (to R.S.R.)
** Fascin crystal structure is available through the RCSB protein data bank. Accession number: 1DFC.

**REFERENCES**

Figure Captions

Fig. 1. **Optically trapped filaments are used to build specific actin architectures for analysis of crosslinker angular dependence of binding.** A, alpha-actinin is active as an anti-parallel homodimer. Each monomer contains an actin binding domain (ABD) comprised of two calponin homology (CH) domains. A series of four spectrin repeats (S1-4) make up the dimerization domain. Each monomer also contains two EF hand domains that are responsible for modulating protein behavior based on calcium signaling (24). Smooth muscle alpha-actinin, the form used in this study, is calcium insensitive. B, fascin is functional as a monomer made of a tight cluster of four fascin domains (F). C, a flow chamber with four lanes is used to add reagents in isolation. Beads are added in channel one, actin filaments in channel two, and crosslinking proteins in channel four. Channel three, which contains only buffer, is used to arrange structures without accidental addition of other components and serves as a barrier between the actin and crosslinker channels to prevent diffusional mixing and unintended aggregation. The black arrows indicate the direction of flow. D, to examine binding behavior of proteins based on actin orientation, two actin filaments were trapped between four 1 µm polystyrene beads. Filaments were crossed and rotated (dashed arrow) until a desired angle was achieved. White arrowheads indicate filament polarity. X’s indicate optical traps. E, once the desired angle was achieved one filament was scanned (dashed arrows) over the other, allowing for the exploration of a large number of potential binding sites. Binding events were recorded when the scanned filament stuck at one point on the stationary filament during the scan and a deformation of the scanned filament was observed. F, parallel and anti-parallel arrangements were also tested. A single filament was stretched out perpendicular to the fluid flow and a second filament was allowed to touch that filament tangentially. If no interaction was observed one of the filaments was rotated 180° and the experiment was repeated. G, to assess if the proteins would bind anti-parallel filaments a single filament was wrapped around a bead and the ends were allowed to interact. Arrows indicate the polarity of the neighboring section of filament. H, addition of a second filament to the wrapped filament assay produces areas of parallel and anti-parallel alignment. For proteins that are selective for parallel versus anti-parallel arrangements, the combination of the assays in G and H clearly shows the preference. I, single bead assays were also performed where two filaments of unknown polarity were attached to a single bead. If they always link, then there is no polarity preference. If they never link then a given protein cannot bind aligned filaments. If they bind 50% of trails it implies that there is a selection for one orientation, the orientation of which a wrapped bead experiment as described in section G can determine.

Fig. 2. **Alpha-actinin is a flexible crosslinker that crosslinks actin in all orientations examined.** A-C, the gallery shows the formation of a single crosslink by alpha-actinin at a 90° cross of two actin filaments on three beads. (Movie S1) D-H, alpha-actinin bundles aligned filaments. The filament marked in red is strung between the two beads and wraps around the top bead. The blue filament is strung between the two beads. All three filament segments bind into a tight bundle. This arrangement necessitates the formation of anti-parallel crosslinks and could include parallel crosslinks as well. (Movie S2) I-P, alpha-actinin crosslink is stable, remaining bound while the link is rotated and pulled in various directions. Gallery shows a 48 second range. This link was observed for more than 3 minutes before the complex fell out of the optical traps. (Movie S3) Scale bar in all figures equals 1 µm.

Fig. 3. **Fascin only crosslinks actin when filaments are arranged in a parallel orientation.** A-C, fascin does not bind in a crossed orientation near 90°. The free end of a filament with only one end bound to a bead was allowed to freely scan over an anchored filament. No binding events were observed. Because of the bend in the filament with both ends anchored to beads, all crossing was near 90° (+/- 10°). Approximately 2.5 µm length of the anchored filament was probed by the free filament end (~900 potential binding sites). At 1 µM fascin concentration ~50% of the available fascin binding sites should be filled, so the scanning filament should have found a viable binding site if the orientation of the filaments was conducive to binding. Dotted lines indicate the range over which the free filament scanned.
(Movie S4) D-I, a pair of crossed filaments were arranged and scanned. No binding events were observed. This pair of filaments was tested over a range of approximately 100° and scanned over a 1.5 μm distance at approximately 15° increments. (Movie S5) J-N, fascin does not bind in an anti-parallel orientation as shown by this filament wrapped around a non-fluorescent bead. Filament ends diffused together, but never remained coupled. (Movie S6) O-S, one filament (red) wraps around a bead that has a second filament (blue) attached. One side of the red filament bundles with the blue filament while the other does not. This behavior is explained by polarity selection. Coupled with the results from J-N we can determine that fascin will bind parallel but not anti-parallel filaments. (Movie S7)

Fig. 4. Bundles do not dissociate even over long timescales. A-F, one long filament was attached in the middle to a bead and the ends were allowed to form a bundle in the presence of 0.1μM alpha-actinin. After bundle formation, the bundle was moved into the buffer lane and all other flow lanes were turned off. The bundle was observed periodically over 40 minutes. The filaments were never observed to separate. (Movie S8) This experiment was replicated six times with the same results, using bundles derived from one to three filaments (two to three lengths of filaments incorporated into the bundle). G-L, two filaments were attached to a bead and the same experiment was performed using 0.1μM Fascin. As with alpha-actinin, the fascin bundles were extremely stable, showing no dissociation over forty minute observations. This experiment was replicated six times using two and three filaments in the bundle. (Movie S9) Additional material on this experiment is found in figures S3 and S4 and movies S10-S13.

Fig. 5. Fluorescence decay and FRAP of labeled fascin in bundles shows the fascin population is stable unless a competitor is added. A-D, bundle of dark actin held together by fluorescent atto-647-fascin (labeled on exposed cysteines using maleimide chemistry, measured 0.95 dyes/fascin) is bleached then observed in a series of movies over 400 seconds. Before bleaching the flow cell was rinsed with buffer to remove free fascin from solution. The boundary between the bleached and unbleached regions of the bundle remained sharp and the signal from the unbleached portion of the bundle remained nearly constant. The white ring indicates the zone of bleaching. Low observational laser power was used to facilitate the long acquisition time by minimizing photobleaching. E, fascin in the bundle is stable in the absence of crosslinker in solution. The graph show the fluorescence decay profile of the four highlighted regions from panel D, recorded during the final movie of this observation. The fluorescence of the bundle (red) remains stable and higher than the other three regions. The green curve is background in the bleached zone, orange is the background outside of the bleached zone and blue is the bundle in the bleached zone. F, fascin in bundles can be competed away. A series of buffer wash experiments using bundles similar to those in panels A-D were performed. In all cases the bundles had been rinsed with buffer to remove free fascin from solution before data was recorded. All data points are background subtracted and then normalized so that a value of 1 corresponded to the average value of the first 25 data points after the wash was initiated. When the bundles were washed with buffer only (red), a slow decay (0.019 ± 0.003 s⁻¹, SEM) was seen in the fluorescent signal. This corresponds to the photobleaching rate at the laser powers used in these experiments. When 3 μM unlabeled fascin (black) was washed in a double exponential decay was observed. The faster rate corresponds to fascin being displaced from the bundle (0.10 ± 0.007 s⁻¹, SEM). This value closely matches that of Aratyn et al., who reported a decay rate of 0.12 s⁻¹. The slower rate (0.023 ± 0.002 s⁻¹, SEM) corresponds to photobleaching. When 3 μM unlabeled alpha-actinin was washed in a rapid single exponential decay was observed (0.254 ± 0.004 s⁻¹, SEM). This is even more rapid than the decay observed with the addition of fascin. This shows that the presence of a competitive agent causes rapid crosslinker turnover. G, fascin replacement is uniform and complete across the entire bundle. With no fascin in solution, a region of a bundle made with fluorescent fascin was bleached. Blue indicates the area of the bundle that was bleached, red indicates the area that was not bleached. After bleaching a significant difference in signal between the two curves is observed. Next 3 μM fluorescent fascin was washed into the chamber (around 20 second
mark, where signal rises to saturation). It was allowed to incubate for 30 seconds and then was washed out with buffer. The wash is completed and signal drops to resolvable levels after approximately an additional 20 seconds. At this point the bundle inside of the bleach zone and outside of the bleach zone have the same fluorescence. This confirms that free fascin can incorporate completely and evenly into the bundle.

**Fig. 6. Models of bundle formation and dynamics.** A, bundles are stable in the absence of competitive agents. Crosslinking proteins that are bound to two filaments (black ovals) stabilize filament bundles. These proteins can toggle to a state (grey ovals) where they are only bound to a single filament. Since the actin site is restrained the single bound crosslinker can readily rebind the second filament. This rebinding rate is faster than the dissociation rate in the single bound state, yielding bundles that are very stable. B, when a competitive agent (white oval) is added to a stable bundle it can occupy actin sites near single bound crosslinkers, preventing their rebinding. This leads to dissociation of the endogenous crosslinker and either replacement with the exogenous agent (shown) or dissociation of both factors (not shown). If crosslinking protein is abundant in solution there is a constant exchange of proteins in the bundle as shown by previous FRAP experiments.
Table 1. **Alpha-actinin binds in all orientations tested.** Alpha-actinin formed crosslinks in all geometries. The first three rows of the table show orientations where the filaments were aligned. In these cases the filaments were linked along the length of the filaments forming bundles. The final three rows show that crossed filaments always crosslinked. The final column shows the probability, using a binomial cumulative distribution function, that only one orientation was sampled during the course of the experiments reported in each row. We did not attempt to control the chirality of filament crosses (eg: 90° with horizontal filament crossed over the vertical or under the vertical) and the probability calculation does not take into account potential chiral differences.

<table>
<thead>
<tr>
<th>Angle (degrees)</th>
<th>Orientation</th>
<th>Orientation Figure Reference</th>
<th>Crosses tested</th>
<th>Crosses that bound</th>
<th>Probability of sampling only one conformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/180</td>
<td>One bead, two filaments</td>
<td>i</td>
<td>6</td>
<td>6</td>
<td>0.0313</td>
</tr>
<tr>
<td>180</td>
<td>One bead, wrapped filament</td>
<td>g</td>
<td>11</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
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<td>Four bead, two filaments</td>
<td>f</td>
<td>5</td>
<td>5</td>
<td>0.0625</td>
</tr>
<tr>
<td>16-45/36-165</td>
<td>Four bead, two crossed filaments</td>
<td>d</td>
<td>7</td>
<td>7</td>
<td>0.0156</td>
</tr>
<tr>
<td>46-89/91-135</td>
<td>Four bead, two crossed filaments</td>
<td>d</td>
<td>3</td>
<td>3</td>
<td>0.25</td>
</tr>
<tr>
<td>90</td>
<td>Four bead, two crossed filaments</td>
<td>d</td>
<td>9</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

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**Table 2. Fascin binding is selective for parallel actin filaments.** Fascin only binds to filaments oriented in a parallel orientation (0°). Crossed filaments (16-165°) form no crosslinks. Filaments oriented in an anti-parallel orientation do not form crosslinks. When two beads are attached to the same filament we observed 8 of 18 tests forming crosslinks. In this orientation approximately fifty percent of the trials should have filaments oriented in a parallel manner and fifty percent in an anti-parallel orientation. When two polarity labeled filaments were allowed to interact in a parallel orientation they were observed to form crosslinks and bundles. From these results we determined that fascin will only form crosslinks when filaments are oriented in a parallel orientation. For crosses of 16°-165° a scan of one angle was performed, then since no crosslinks were observed a new angle was selected and another scan was performed with that same filament pair. The notation 4/4 in the crosses tested field mean four crosses were formed, and each of those crosses was tested in both orientations represented in that bin on the table. The same meaning is intended for the 5/5 notation.

<table>
<thead>
<tr>
<th>Angle (degrees)</th>
<th>Orientation</th>
<th>Orientation Figure Reference</th>
<th>Crosses tested</th>
<th>Crosses that bound</th>
<th>Probability of sampling only one conformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Polarity labeled, four bead, two filaments</td>
<td>1f</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>180</td>
<td>Polarity labeled, four bead, two filaments</td>
<td>1f</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0/180</td>
<td>One bead, two filaments</td>
<td>1i</td>
<td>18</td>
<td>8</td>
<td>7.6 x10^-6</td>
</tr>
<tr>
<td>180</td>
<td>One bead, wrapped filament</td>
<td>1g</td>
<td>9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>16-45/36-165</td>
<td>Four bead, two crossed filaments</td>
<td>1d</td>
<td>4/4</td>
<td>0</td>
<td>N/A – four crosses rotated through range</td>
</tr>
<tr>
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<td>1d</td>
<td>5/5</td>
<td>0</td>
<td>N/A – five crosses rotated through range</td>
</tr>
<tr>
<td>90</td>
<td>Four bead, two crossed filaments</td>
<td>1d</td>
<td>5</td>
<td>0</td>
<td>1</td>
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</tbody>
</table>
Figure 3
Figure 5

Prebleach

Fluorescence

Time (sec) Post-bleach

0s 150s 360s

E

Fluorescence

Time (sec) Post-bleach

F

Normalized Fluorescence

Time (sec) Post-bleach

G

Fluorescence

Time (sec) Post-bleach